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Calcium/Phosphatidylserine/Diacylglycerol-dependent Protein Phosphorylation in the *Aplysia* Nervous System[1]

SUSAN A. DeRIEMER,*[,2] PAUL GREENGARD,‡ AND LEONARD K. KACZMAREK*[,3]

* *Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510 and* ‡ *The Rockefeller University, New York, New York 10021*

Abstract

It has been shown that intracellular injection of protein kinase C (calcium/phosphatidylserine/diacylglycerol-dependent protein kinase), purified from mammalian brain, or application of the tumor-promoting phorbol diester, 12-*O*-tetradecanoyl-13-phorbol acetate (TPA), leads to an enhancement of calcium currents in the bag cell neurons of *Aplysia*. We now present evidence of an endogenous enzyme in bag cell neurons which is activated by TPA and which has properties similar to those of mammalian protein kinase C.

Calcium/phosphatidylserine/diacylglyerol-dependent protein kinase activity was found in both cytosolic and particulate fractions prepared from isolated clusters of bag cell neurons. This endogenous enzyme phosphorylated an 87,000-dalton protein from bovine brain, which appears to be a specific substrate for protein kinase C, as well as several substrates present in cytosolic fractions prepared from isolated bag cell clusters. Similar results were obtained using preparations made from pooled head ganglia from *Aplysia*.

The pharmacological properties of the calcium/phosphatidylserine/diacylglycerol-dependent protein kinase activity in the *Aplysia* nervous system were similar to those of protein kinase C from mammalian tissues. Thus, the same group of endogenous substrate proteins were phosphorylated when diacylglycerol was replaced by TPA in cytosolic fractions prepared from isolated bag cell clusters. Non-tumor-promoting phorbols (4-α-phorbol, 4-α-phorbol-12,13-didecanoate, and 4-*O*-methyl-12-*O*-tetradecanoylphorbol-13-acetate) did not stimulate protein phosphorylation in these preparations. Phosphorylation by the *Aplysia* calcium/phosphatidylserine/diacylglycerol-dependent protein kinase was inhibited by polymixin B sulfate, by calmodulin, and by the "calmodulin antagonists" trifluoperazine, calmidazolium and W7.

The presence of calcium/phosphatidylserine/diacylglycerol-dependent protein kinase activity in *Aplysia* neurons, together with the effectiveness of protein kinase C in regulating calcium channels in these cells, strongly implicates this enzyme in the regulation of neuronal excitability.

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[2] Present address: Max Planck Institut fur Biophysikalische Chemie, Postfach 2841, D-3400 Goettingen, Federal Republic of Germany.

[3] To whom reprint requests should be addressed.

Protein kinase C is a calcium/phosphatidylserine/diacylglycerol-regulated protein kinase. It is present in high concentrations in the mammalian brain, and there is evidence suggesting that it is involved in synaptic function. Protein kinase C can be stimulated by diacylglycerol, one of the products generated by neurotransmitter-stimulated phosphatidylinositol turnover (Takai et al., 1979b). In non-neuronal cells, activation of protein kinase C may be associated with secretion (Nishizuka, 1984). Protein kinase C activity in brain copurifies with the major binding protein for phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Shoyab and Todaro, 1980; Leach et al., 1983; Niedel et al., 1983). Tumor-promoting phorbol esters have been shown to activate protein kinase C (Castagna et al., 1982; Kikkawa et al., 1983; Yamanishi et al., 1983) and to influence neurotransmission (Baraban et al. 1984). Phorbol ester-binding sites in the mammalian brain appear concomitantly with synaptogenesis during development and are concentrated in regions rich in synapses in the adult brain (Murphy et al., 1983).

Because of the relative simplicity of molluscan nervous systems and the large size of individual cells, it is possible to characterize the effects of intracellular enzymes on neuronal activity and synaptic transmission to an extent not yet possible with mammalian cells. In our studies we have used the bag cell neurons of *Aplysia*, which are an anatomically discrete, homogeneous population of neurons located within the abdominal ganglion in two clusters of about 400 neurons each. In response to appropriate stimuli, they undergo a series of long-lasting changes in their electrical properties (Kupfermann and Kandel, 1970), including a 30-min afterdischarge and a 20-hr refractory period, during which it is known that the phosphorylation state of specific proteins is altered (Jennings et al., 1982). At least some of these changes are believed to require changes in intracellular calcium (Kaczmarek et al., 1982; Kaczmarek and Kauer, 1983). The cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinases present in these cells have previously been characterized, and a role for cAMP-mediated protein phosphorylation in the regulation of potassium channels has been found (Jennings et al., 1982; Kaczmarek et al., 1980; DeRiemer et al., 1984a).

The analysis of the role of protein kinase C in these neurons was carried out in two stages. The first, presented here, consisted of the biochemical and pharmacological characterization of protein kinase C in the bag cell neurons and was designed to address the following questions: (*1*) Is protein kinase C present in the bag cell neurons? (*2*) Is the *Aplysia* enzyme similar to the enzyme which has been purified from mammalian brain? (*3*) Is *Aplysia* protein kinase C activated by phorbol esters? and (*4*) Are there drugs which inhibit this enzyme? Although calcium/phosphatidylserine/diacylglycerol-dependent protein phosphorylation has been observed in nervous tissue from a number of phyla, including molluscs (Takai et al., 1979a; Kuo et al., 1980; Kikkawa et al., 1982), a detailed comparison of enzyme activity from mammalian and non-mammalian sources

has not yet been carried out. The similarity described here between *Aplysia* protein kinase C and protein kinase C which has been purified from vertebrate brain suggested that intracellular injections of the vertebrate enzyme as well as its pharmacological activation could be used to manipulate the activity of this enzyme within bag cell neurons while monitoring the activity of ion channels. This second stage, presented elsewhere (DeRiemer et al., 1984b, 1985), used these techniques to show that protein kinase C can modulate the calcium current in the bag cell neurons.

Materials and Methods

Aplysia californica (Alacrity Marine Biological Services, Newport Beach, CA) was maintained in artificial seawater (Instant Ocean) at 14°C. Animals were sacrificed, and the central nervous system ganglia were removed and cleared of as much connective tissue as possible. Isolated bag cell clusters were dissected away from the remainder of the abdominal ganglion. The tissue was homogenized on ice in 0.2 ml (bag cell cluster) or 1.0 ml (pooled head ganglia) of homogenization buffer (50 mM Tris, pH 7.2, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM leupeptin, and 50 units/ml of Trasylol) using a ground glass tissue grinder. Excess connective tissue was removed by centrifugation at 1,000 × *g* for 1 min. Protein was determined by the method of Lowry et al. (1951). Particulate and cytosolic fractions were prepared by centrifugation of homogenates at 100,000 × *g* for 1 hr in a Beckman ultracentrifuge. Pellets were resuspended in the original volume of homogenization buffer.

Standard phosphorylation assays were carried out in a final volume of 100 μl containing 50 μl of the appropriate tissue fraction (0.2 to 1.0 mg/ml of protein), 10 mM $MgCl_2$, 5 μg/ml of cAMP-dependent protein kinase inhibitor protein, and the indicated additions. For calcium-free solutions, EGTA was added to a final concentration of 1.5 mM. Where indicated, calcium, phosphatidylserine, and 1,3-diolein were added to final concentrations of 1.0 mM, 50 μg/ml, and 5 μg/ml, respectively. These concentrations of phosphatidylserine and diolein have been used in studies of mammalian protein kinase C and stimulate phosphorylation in preparations of the *Aplysia* nervous system containing the range of protein concentrations given above. Solutions of phosphatidylserine and diolein were sonicated immediately before addition. Reaction mixtures were preincubated for 1 min at 20°C and reactions were initiated by addition of [γ-^{32}P]ATP (50 μM). Reactions were stopped after 2 min by addition of 50 μl of "stop" solution (20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 9% (w/v) SDS, 125 mM Tris, pH 6.8, 3 mM EDTA and a trace of bromphenyl blue) immediately followed by boiling for 2 min. Samples were subjected to SDS-PAGE using the system of Laemmli (1970). Gels were stained for protein with Coomassie brilliant blue, destained, dried, and autoradiographed using Kodak X-Omat film and DuPont Lightning Plus intensifying screens. Phosphate incorporation into specific protein bands was quantitated by cutting the bands from the dried gel, followed by liquid scintillation counting.

The 87,000-dalton substrate for protein kinase C, purified from bovine brain by the method described by Albert et al. (1984) was a generous gift of K. Albert. Calmodulin, purified by the method of Grand et al. (1979), was a gift of Y. Lai. The inhibitor protein of the cAMP-dependent protein kinase (Ashby and Walsh, 1972) was a gift of Dr. A. Nairn. [γ-^{32}P]ATP was obtained from Amersham Corp (Arlington Heights, IL) and diluted with cold ATP to 5 to 10 × 10^7 cpm/nmol. Calmidazolium (R24571; 1-[bis(*p*-chlorophenyl)-methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]imidizolium chloride) was obtained from Janssen Pharmaceuticals (Beerse, Belgium). Trifluoperazine (TFP) was obtained from Smith, Kline and French. W7 (*N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide hydrochloride) was obtained from Rikaken Co., Ltd. (Nagoya, Japan). Polymixin B sulfate, phosphatidylserine, 1,3-diolein, histone H1, leupeptin, PMSF, and molecular weight markers were obtained from Sigma. The molecular weight markers used were: myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200).

Results and Discussion

Calcium/phosphatidylserine/diacylglycerol-dependent protein phosphorylation. Calcium/phosphatidylserine/diacylglycerol-de-

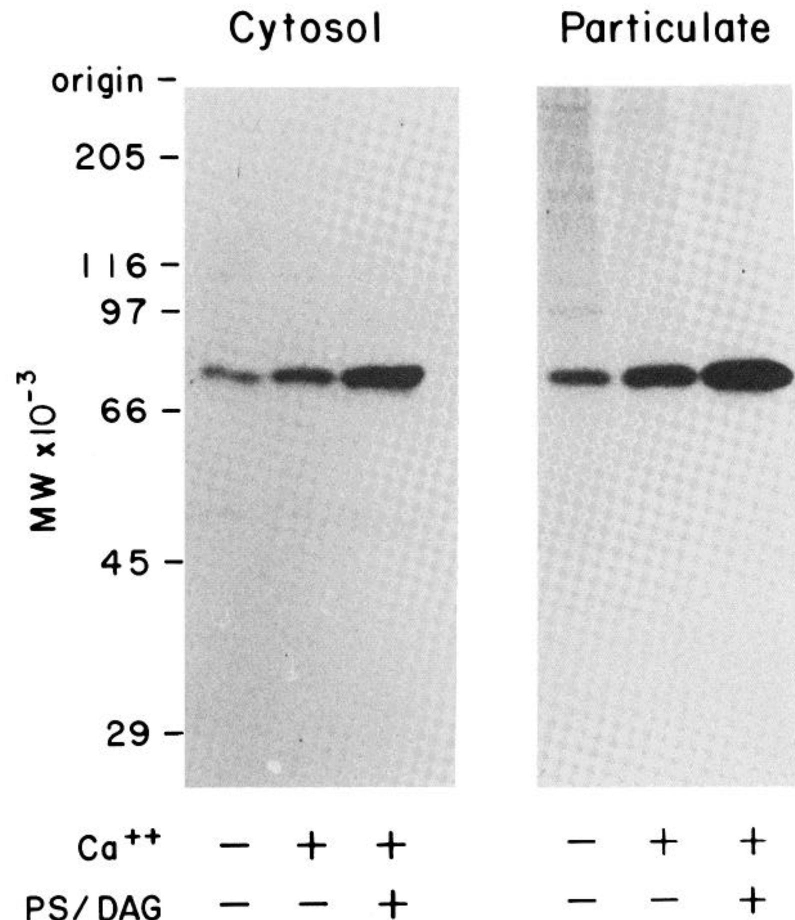

Figure 1. Phosphorylation of a substrate protein, specific for protein kinase C, by both particulate and cytosolic *Aplysia* protein kinase C. The 87,000-dalton protein from bovine brain, a specific substrate for protein kinase C, was phosphorylated by endogenous enzymes in a 100,000 × *g* supernatant (*Cytosol*) or pellet (*Particulate*) fraction prepared from isolated bag cell clusters. Proteins were phosphorylated in the presence of [γ-^{32}P]ATP in the absence (−) or presence (+) of calcium (*Ca*$^{++}$; 1 mM), phosphatidylserine (*PS*; 50 μg/ml), or 1,3-diolein (*DAG*; 5 μg/ml) as indicated. Radioactive proteins were separated by SDS-PAGE and detected by autoradiography. The exposure of this autoradiogram was adjusted for the 87,000-dalton phosphoprotein such that the phosphorylation of endogenous proteins is not visible. The 87,000-dalton phosphoprotein is indicated by the *arrowhead*. In this experiment, addition of calcium/phosphatidylserine/diacylglycerol stimulated phosphate incorporation 2- to 3-fold over that seen in the presence of calcium alone.

pendent phosphorylation was assayed using an exogenous 87,000-dalton protein purified from bovine brain (Wu et al., 1982). This protein appears to be a specific substrate for mammalian brain protein kinase C and is not phosphorylated by either the cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinases (Wu et al., 1982; J. Wang, S. I. Walaas, and P. Greengard, unpublished observations). Calcium/phosphatidylserine/diacylglycerol-stimulated phosphorylation was observed in both the cytosolic and particulate fractions prepared from bag cell neurons (Fig. 1). Similar results were obtained using a second known substrate for protein kinase C, histone H1. Calcium/phosphatidylserine/diacylglycerol-dependent phosphorylation of the 87,000-dalton protein and of histone H1 was also observed using either particulate or cytosolic fractions prepared from *Aplysia* head ganglia (data not shown).

Calcium/phosphatidylserine/diacylglycerol-dependent phosphorylation of several endogenous substrate proteins was observed in cytosolic fractions prepared from isolated bag cell clusters (Fig. 2) or head ganglia (data not shown). These endogenous substrate proteins had M_r values of 215,000, 157,000, 134,000, 121,000, 105,000, 87,000, 70,000, 56,000, 44,000, 38,000, 33,000, 28,000, and 16,500. No differences were observed in the substrates present in isolated bag cell clusters and those in head ganglia. In these fractions the addition of calcium alone or the addition of phosphatidylserine plus diolein in the absence of calcium had little or no effect on phosphorylation. It was not possible to observe calcium/phosphatidylserine/diacylglycerol-dependent phosphorylation of endogenous substrates in homogenates or in particulate fractions prepared from bag cell neurons or head ganglia because of the high level of phosphorylation observed upon addition of calcium alone (in part due to calcium/calmodulin-dependent protein phosphorylation in such preparations).

Pharmacological properties of calcium/phosphatidylserine/diacylglycerol-dependent protein kinase in Aplysia neurons. Calcium/phosphatidylserine/diacylglycerol-dependent phosphorylation of several endogenous substrates could be inhibited by a number of drugs in cytosolic fractions prepared from *Aplysia* head ganglia (Fig. 3). Three of the drugs tested are commonly referred to as "calmodulin antagonists" (TFP, calmidazolium (R24571), and W7). Trifluoperazine and several other phenothiazines, however, were previously shown to inhibit protein kinase C (Mori et al., 1980; Schatzman et al., 1981). The fourth drug, polymixin B, has been reported to show some selectivity in inhibiting protein kinase C (Kuo et al., 1983). Trifluoperazine and W7 inhibited the activity of the *Aplysia* calcium/phosphatidylserine/diacylglycerol-dependent protein kinase (Fig. 3) at approximately the same doses that block calcium/calmodulin-dependent phosphorylation (DeRiemer et al., 1984a) in these cells. Calmidazolium, in contrast, was approximately 10 times less effective against *Aplysia* protein kinase C (Fig. 3) than against the calcium/calmodulin-dependent protein kinase activity. In general, it appears that these drugs cannot be used as specific antagonists of either protein kinase C or calmodulin-dependent enzymes.

Calmodulin can inhibit protein kinase C purified from mammalian brain (Albert et al., 1984). The results shown in Figure 4 indicate that exogenously added calmodulin is capable of inhibiting the calcium/phosphatidylserine/diacylglycerol-stimulated phosphorylation of the 87,000-dalton protein by the particulate fraction from *Aplysia* head ganglia. Similar results were obtained using the cytosolic fraction from these ganglia although these showed more variability, most likely due to the presence of endogenous calmodulin.

The phorbol ester TPA, originally described as a tumor promoter (Hecker, 1968), has been shown to stimulate the activity of protein kinase C (Castagna et al., 1982; Yamanishi et al., 1983). The ability of TPA and phorbols that lack tumor-promoting activity (4-*O*-methyl-12-*O*-tetradecanoylphorbol-13-acetate, 4-α-phorbol, and 4-α-phorbol-12,13 didecanoate) to stimulate phosphorylation in pooled head ganglia from *Aplysia* was examined. In the presence of phosphatidylserine, TPA stimulated phosphate incorporation into exogenously added 87,000-dalton protein. None of the inactive derivatives stimulated phosphorylation, even when applied at twice the maximum dose tested for TPA (Table I). The pattern of endogenous substrate proteins phosphorylated by cytosolic fractions prepared from either isolated bag cell neurons (Fig. 2, *lane D*) or head ganglia (data not shown) was similar when TPA was used instead of 1,3-diolein to stimulate phosphorylation. This similarity suggests that the TPA-stimulated phosphorylation is due primarily to the activation of protein kinase C in these cells.

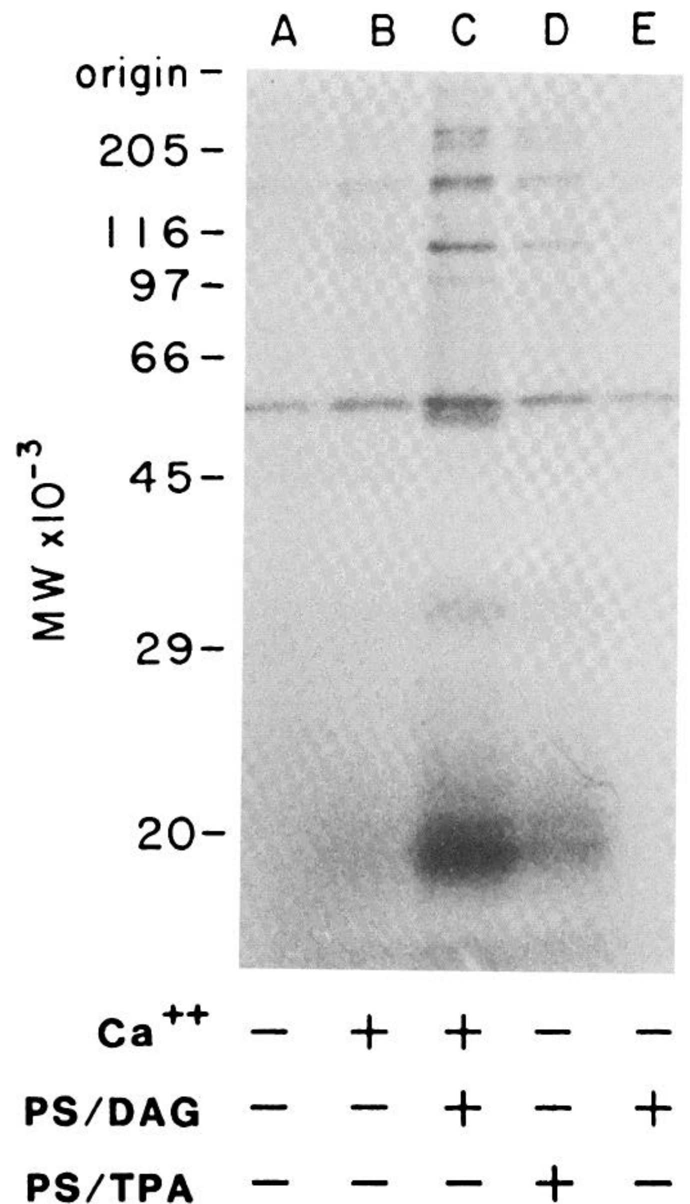

Figure 2. Phosphorylation of endogenous substrate proteins by *Aplysia* protein kinase C. Cytosolic fractions were prepared from isolated bag cell clusters. Proteins were phosphorylated in the presence of [γ-^{32}P]ATP in the absence (−) or presence (+) of calcium (Ca^{++}; 1 mM), phosphatidylserine (*PS*; 50 μg/ml), 1,3-diolein (*DAG*; 5 μg/ml), and TPA (100 nM) as indicated. Radioactive proteins were separated by SDS-PAGE and detected by autoradiography.

Physiological considerations. In the present investigation we have characterized a calcium/phosphatidylserine/diacylglycerol-dependent protein kinase in molluscan neurons. This enzyme resembles its mammalian counterpart with respect to subcellular localization (Kikkawa et al., 1982), its ability to phosphorylate a specific substrate protein from vertebrate brain (Wu et al., 1982) as well as histone H1

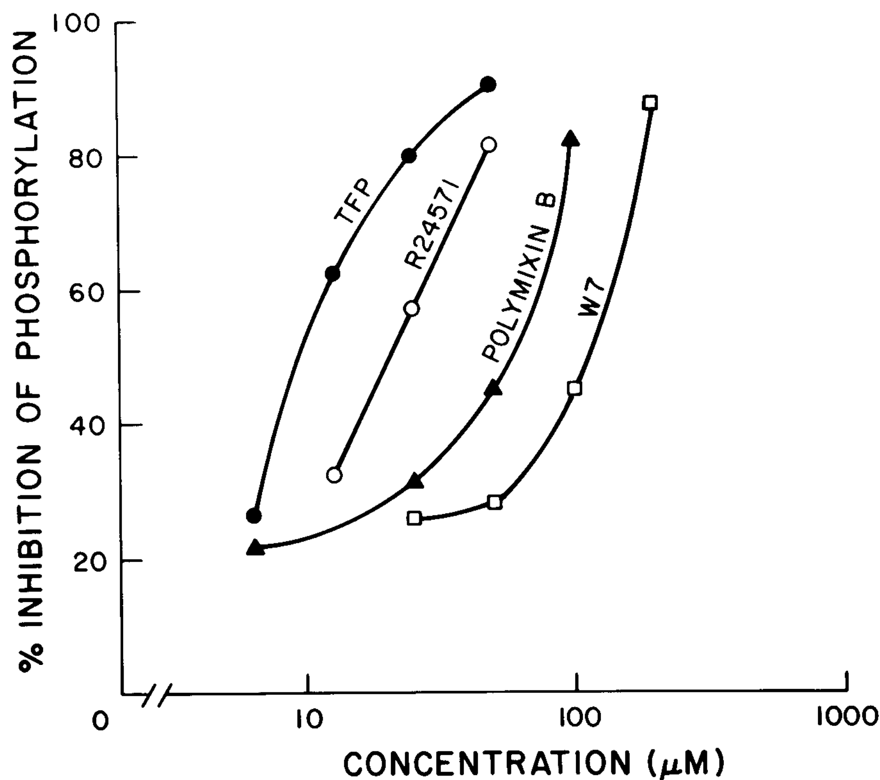

Figure 3. Inhibition of *Aplysia* protein kinase C. Protein kinase C activity in cytosolic fraction prepared from *Aplysia* head ganglia was quantitated by measuring phosphate incorporation into the 16,500-dalton substrate protein; similar results were obtained when two other endogenous substrates, the 157,000- and 215,000-dalton phosphoproteins, were used for quantitation. Proteins were phosphorylated in the presence of [γ-^{32}P]ATP, calcium (1 mM), phosphatidylserine (50 μg/ml), and 1,3-diolein (5 μg/ml). TFP, calmidazolium (*R24571*), polymixin B sulfate, or W7 was present in the final concentrations indicated. Data represent the means of three experiments.

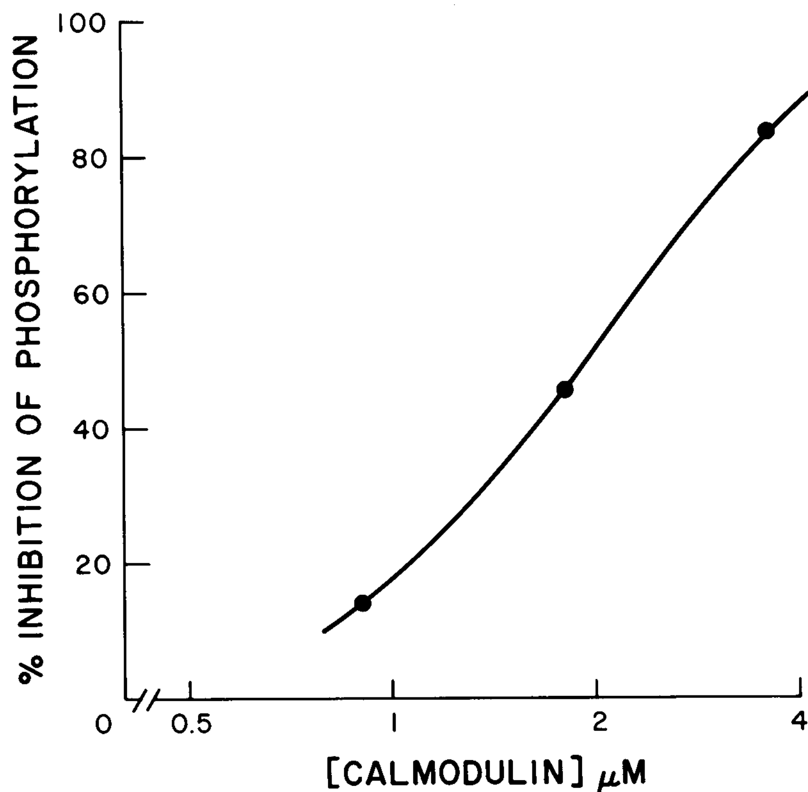

Figure 4. Inhibition by calmodulin of *Aplysia* protein kinase C. Phosphate incorporation into the 87,000-dalton protein from bovine brain, a specific substrate for protein kinase C, was assayed in a particulate fraction prepared from *Aplysia* head ganglia in the presence of calcium (1 mM), phosphatidylserine (50 μg/ml), 1,3-diolein (5 μg/ml), and exogenous calmodulin at the final concentrations indicated.

(Takai et al., 1979b), inhibition by polymixin B (Kuo et al., 1983), TPF (Schatzman et al., 1981), and calmodulin (Albert et al., 1984), and its stimulation by TPA (Castagna et al., 1982; Yamanishi et al., 1983). Electrophysiological evidence presented elsewhere has demonstrated that either application of TPA or intracellular injection of protein kinase C enhances calcium action potentials and that TPA acts by increasing a voltage-dependent calcium current in *Aplysia* bag cell neurons. Considered together, these studies strongly suggest that activation of protein kinase C in *Aplysia* bag cell neurons, resulting in increased phosphorylation of endogenous substrate protein(s), represents part of the molecular machinery by which these neurons respond to extracellular stimuli with changes in their electrical properties.

Within the *Aplysia* nervous system and, in particular, within the

TABLE I

Stimulation of Aplysia protein kinase C by phorbols

Phosphate incorporation into the 87,000-dalton bovine substrate for protein kinase C was assayed in cytosolic fractions prepared from pooled head ganglia in the presence of phosphatidylserine (50 μg/ml), 1.5 mM EGTA, and the indicated agents. Data are expressed as percentage of change relative to phosphate incorporation in the absence of added agents and represent the means and SEM from three experiments. The effects of adding calcium (Ca; 1 mM) plus 1,3-diolein (DAG; 5 μg/ml) is also shown (n = 6).

Phorbol	Concentration (nM)	Phosphorylation (% ± SEM)
TPA	50	49.5 ± 5.1
	200	77.0 ± 33.6
4-α-Phorbol	400	−36.1 ± 11.1
4-α-Phorbol-12,13-didecanoate	400	−15.7 ± 6.3
4-*O*-Methyl-12-*O*-tetradecanoyl-phorbol-13-acetate	400	−5.9 ± 23.8
Ca/DAG		58.7 ± 23.3

bag cell neurons, protein kinase C coexists with at least two other protein kinase systems, cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase(s) (Jennings et al. 1982; Novak-Hofer and Levitan, 1983; DeRiemer et al., 1984a). The increased calcium current resulting from activation of protein kinase C (DeRiemer et al. 1984b, 1985) and the inhibition of potassium currents produced by cAMP-dependent protein phosphorylation (Kaczmarek and Strumwasser, 1984; Strong, 1984; Kaczmarek et al., 1985) provide these cells with two separate pathways which can transform the electrical properties of these neurons in the direction of increased excitability. This contrasts with the situation in platelets, a non-neuronal system in which the actions of protein kinase C have been well characterized, in which the actions of cAMP and protein kinase C appear to be antagonistic (Nishizuka, 1984).

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